PHYSIOLOGY OF RESPIRATORY SYSTEM and
CHRONIC PULMONARY DISEASES

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Act: 1/1/70
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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

COMMITTEE:
Dr. Loosli
Dr. Meier
Dr. Sommers

Application For Research Grant

Date: July 26, 1972.

1. Name of Investigator(s): (include Title and Degrees)

2. Institution & Address:

- Lauweryns, Joseph-M., M.D., Ph.D., Professor ordinarius in Microscopic Anatomy and Pathology ; Chairman, Principal Investigator.
- Boussauw, Luc, Lic. Biol. Sc., full-time research assistant, doctorandus. Co-investigator.
- Desmecht, Monique (Mrs. Gombeer), Lic. Biol. Sc., full-time research assistant. Co-investigator.

Experimental Laboratory of Cardiopulmonary and Genital Pathology, Department of Pathology, University of Leuven, 12, Minderbroedersstraat, B - 3000 LEUVEN-BELGIUM.

3. Short Title of Project:

The lymphatics of the lung. Their role in fluid transport and clearance of airborne particulate matter in normal and experimental conditions and in various lung diseases.

4. Proposed Starting Date:

January 1, 1973.

5. Anticipated Duration of this Specific Study:

Three (3) years.

6. Brief Descripton of Objectives or Specific Aims:

Despite our experience in the proposed field of investigation, numerous aspects of the structure and function of the pulmonary lymphatics are still either largely unknown or a matter of considerable controversy in the literature. Each technique of study having its shortcomings, these can only be solved accurately by a further and multidisciplinary investigation of the pulmonary lymphatics which will include various techniques, i.e. - anatomical injection studies, - stereomicroscopical studies, - serial reconstructions, - radiography and microradiography, - histological techniques, - morphometrical techniques, - histochemical techniques, - transmission electron microscopy, - freeze-etching electron microscopy and scanning electron microscopy.

During the three years of this research proposal we intend to study the pulmonary lymphatics - with the techniques proposed - along three major lines of investigation : - (1) normal morphology of the pulmonary lymphatics, - (2) an experimental study of the various morphological factors involved in the clearance and lymphatic drainage of the lung parenchyma, - (3) and morphological studies of the lung lymphatics in various pathological conditions especially in such cases where the formation of lung edema is observed (e.g. neonatal lungs : hyaline membrane disease ; adult lungs : pulmonary edema, especially when associated with chronic respiratory insufficiency as in chronic bronchitis or emphysema, shock lungs (cardiogenic and neurogenic), uremia, drowning...). Though the lines of investigation are distinct, the study object (i.e. the lymphatics) is identical and the results interrelated.

These studies will necessarily end in important and original contributions which will have many basic and applied results in the structure and function of the normal and diseased lung. It is obvious that these studies are of immediate and relevant importance in biological tobacco research.

7. Give a Brief Statement of your Working Hypothesis:

A combined and multidisciplinary morphological investigation by a team of investigators working closely together since several years, will allow to avoid the shortcomings of each individual technique of investigation in lymphatic research. Studies of pulmonary fluid transport and of airborne particulate matter in normal and diseased lungs and in experimental conditions urgently request a precise and up-to-date knowledge of the pulmonary lymphatics, which is still lacking.

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See separate pages - Addendum 1.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

- All physical facilities are available, except item 10. Additional requirements.
- Separate list of these physical facilities - see addendum 2.

10. Additional Requirements: One scanning electron microscope.

See separate page : Addendum 3.

Biographical sketches of all principal and professional personnel (append)

See separate pages - Addendum 4

12. List of publications: (Five most recent as pertinent) (append) and Progress Report (January 1970 - July 1972).

See separate pages - Addendum 5.

13. Budget (1st year) (In U.S. Dollars)

A. Salaries (Personnel by names)	% time	Amount
Professional		
Technical		
Two laboratory technicians	100%	10,830
Sub-Total		10,830
B. Consumable Supplies (list by categories)		
animals		2,260
supplies		8,000
Sub-Total		10,260
C. Other Expenses (itemize)		
		none
Sub-Total		none
D. Permanent Equipment (itemize)		
First part-payment (= one sixth of its cost price) of a scanning electron microscope Philips, to be paid in three years - see item 10. Additional requirements - Addendum 3		17,716
		none
E. Overhead (15% of A+B+C)		
Total		38,806

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	11.080	10.510		17.716	none	39,306
Year 3	11.330	10.760		17.716	none	39,806

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature H. Toly Lauwerys
Director of Project

Belgium (016) 28981
Telephone

Signature
Business Officer of the Institution
P. DE SOMER
Rector of the University

Telephone

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

	Title of Project	Source	Amount	Duration
Current	Morphological studies of the Lung.	University of Leuven J.M. Lauweryns, professor, salary L. Boussauw, assistant, salary M. Desmecht, assistant, salary Salary of two technicians	$ 12,000 $ 6,500 $ 6,500 $ 11,000	yearly " " "
		Supplies and animals	$ 5,000	"
	Same area of interest since 9 years	Equipment - as listed under "physical facilities available" - from grants and local university.	This equipment will be available.	
Pending	Same project as now submitted to CTR	University of Leuven Matching grant of the University of Leuven, defraying 50% of the purchase of the requested Scanning Electron Microscope, if CTR approves our current research proposal i.e.		
		1973 : first part (one sixth) 1974 : second part (one sixth) 1975 : third part (one sixth)	$ 17,716 $ 17,716 $ 17,716	

ADDENDUM 1

8. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES

THE LYMPHATICS OF THE LUNG. THEIR ROLE IN FLUID TRANSPORT AND CLEARANCE OF AIRBORNE PARTICULATE MATTER IN NORMAL AND EXPERIMENTAL CONDITIONS AND IN VARIOUS LUNG DISEASES

DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES. RESEARCH PLAN

A. INTRODUCTION AND SPECIFIC AIMS

(1) Literature

1.1. As regards the normal morphology of the pulmonary lymphatic system a considerable interest has developed in recent years and much time and efforts have been spent in the study of its structure and function. Especially in the morphological field and following injection studies (Lauweryns et al., publication nr. (26), 1962, (38), 1965, (69), 1970, (89), 1971, Pennell, 1966, Pump, 1970, Trapnell, 1963, Bastianini, 1967, a,b), histological (Lauweryns et al., publication nr. (31), 1963, (34), 1964, (36), 1964, (37), 1965, (46), 1966, (60), 1968, (85), 1970, (86), 1970, (121), 1972, Aminova, 1963,1967, Grau, 1965, Jdanov, 1969, Karpe, 1965, Kriz, 1970, Oehmke, 1968), histochemical (Borst et al., 1969, Fruschelli, 1967, 1966), radiological (Pennell, 1966, Trapnell, 1963, Lauweryns et al., publication nr. (26), 1962, (34), 1964, (69), 1970, (96), 1971) and especially electron microscopic (Borst et al., 1969, Lauweryns et al., publication nr. (57), 1968, (88), 1970, (96), 1971, (115), 1971, (124), 1972, (125), 1972, Casley-Smith, 1961,1967, Cliff, 1970, Collan et al., 1970, Fruschelli, 1970, Kato, 1966 a, 1966 b, Klika, 1968, Kriz, 1970, Kühnel, 1966, Leak, 1966, 1967, 1968, 1968, 1970, Oehmke, 1968, Schipp, 1967, 1968, Takada, 1971, Vajda, 1971, Viragh, 1966) investigations of the lymphatic vessels of several body regions, important discoveries have been made, which have led in turn to new ideas and hypothesis concerning the functioning of the lymphatic system. In this way the existence of open junctions (Leak, 1965, 1966, 1968, 1968, 1971, Casley-Smith, 1961, 1967, 1969) and of anchoring filaments (Leak, 1966, 1967, 1968) led to the flap valve concept of the endothelial junctions (Collan, 1970). The absence of a continuous basement membrane and the presence of open junctions and cytoplasmic vesicles (Casley-Smith, 1969) explained the high permeability of the lymphatic endothelium. The presence of filaments within the lymphatic endothelial cells suggests that these cells might have an active and contractile role (Majno, 1969, Schipp, 1968). The recovery of important amounts of tracer proteins (Leak, 1970, 1971, Földi, 1955) in the lymphatic endothelium suggests that these cells might have phagocytotic properties. The ultrastructural aspect and composition of lymphatic valves also raised important ideas about their functioning (Lauweryns et al., (85), 1970, (104), 1970, (96), 1971, (115), 1971, Vajda and Tomcsik, 1971).

Our own investigations were directed almost exclusively towards the lymphatics of the lung for several reasons. First of all, we were struck by the contradiction between the idea that the pulmonary tissue was a "dry" tissue in which no lymph was formed, as a consequence of the low pressure of the pulmonary circulation (Földi, 1964) on the one hand, and the very extensive pulmonary lymphatic plexus on the other hand (Lauweryns, publication nr. (85), 1970, (115), 1971). Secondly we realized the importance of the pulmonary lymphatic system at birth, when the fluid contents of the pulmonary airways and alveoli is apparently largely and very quickly removed via the pulmonary lymphatics (Aherne and Dawkins, 1964, Boston, 1965, Humphreys et al., 1967). Failure of this removal is somehow related to the hyaline membrane disease (Lauweryns et al., publication nr. (38), 1965, (39), 1965, (56), 1968, (70), 1968, (74), 1968, (76), 1968, (80), 1969, (88), 1970) or the idiopathic respiratory distress syndrome of the newborn, a disease responsible for a large percentage of neonatal deaths (Avery, 1964). Finally the importance of the pulmonary lymphatic system for the efficiency and well functioning of respiration and vice versa is gradually becoming clear (Maier, 1966).

Our efforts were successful and we were able to extend the knowledge obtained mainly from lymphatics from skin and mesenterium (Borst, 1969, Casley-Smith, 1961, 1967, Cliff, 1970, Leak and Burke, 1968, Oehmke, 1968, Ohkuma, 1970, Schipp, 1968) and to compare them to the pulmonary lymphatics ; to confirm various aspects of their structure and to contribute with several important discoveries, such as the juxta-alveolar lymphatics and the pericentriolar filamentous bundles (Lauweryns et al., publication nr. (38), 1965, (56), 1968, (57), 1968, (60), 1968, (69), 1970), (70), 1968, (74), 1968, (76), 1968, (80), 1969, (85), 1970, (86), 1970, (104), 1970, (121), 1972, (124), (125), Lauweryns, publication nr. (26), 1962, (31), 1963, (34), 1964, (36), 1964, (37), 1965, (39), 1965, (46), 1966, (88), 1970, (89), 1971, (96), 1971, (115), 1971).

1.2. As regards an experimental approach of the various morphological factors involved in the clearance and drainage of the lung parenchyma, we have summarized the available data of the literature on the hereby included scheme 1.

A simple glance at this scheme[1] reveals that :

- no morphological data are available on the precise mechanisms of lymphatic lung drainage (except for the light optical tracer studies of Casarett et al., 1964), and that

- much contradiction exists as to the results obtained, e.g. in the all or not fagocytotic properties of the type I and type II alveolar epithelial cells, even when the investigators have used the same tracer substance (see scheme 1 for thorotrast, carbon and chinese India Ink studies).

SCHEME I

AUTHORS	MORPH. INVEST.	ANIMAL	TRACER	WAY OF ADMINISTRATION	TIME INT.	P.ALV. WALL	ALV. MACR.	LEUKOC.
DRINKER e.a. 1937	-	dog	serum protein haemoglobin egg-albumin	intratracheal injection		+ + +		
JRTICE and SIMMONDS, 1949	-	rabbit	albumin	intratracheal instillation	9 h.			
SWANN and SPAFFORD, 1951	-	dog	various ions	intratracheal instillation		+ exc.sulf		
GROSS and WESTRICK, 1954	+ (LO)	rat	india ink	intratracheal injection	4 h.à4 d.	+	+	+
LOW and SAMPAIO, 1957	+ (EM)	rat	thorotrast	intratracheal instillation	1/2 h.		+	
GIESEKING, 1958	+ (EM)	rat	ferric-hydrox carbon gold haemoglobin india ink	intratracheal injection	10 min. to 8 w.		+ + + + +	+ + + + +
KARRER, 1958	+ (EM)	mouse	india ink	intranasal instillation	1 1/2 h.		+	
POLICARD e.a. 1959	+ (EM)	rat	silicium	intratracheal injection			+	
KARRER, 1960	+ (EM)	mouse	india ink	intranasal instillation	2 h.à 1d. 9 d.		+	+
SCHULTZ e.a. 63	-	dog	I^{131} albumin-I^{131}	intrabronchial instillation	1m. à144h 1m. à144h	+ +		
CASARETT, 1964	+ (LO)	rat	Polonium-210	inhalation			+	
CASARETT and MILLEY, 1964	+ (LO)	rabbit	$^{210}Po(OH)_2$ / $^{239}PuO_2$	inhalation			+	
CASARETT and MORROW, 1964	+ (LO)	rabbit	$^{210}Po(OH)_2$ ^{210}Polonium tagged silver	intratracheal instillation	1 à 30 d. 1 à 28 d.		+ +	
SCHULTZ e.a. 1964	-	isolated lung of dog	albumin-I^{131}	intrabronchial instillation	1m.à 5 h. 1h.à 24 h			
LADMAN and FINLEY, 1966	+ (EM)	dog	thorotrast	incubation of "alveolar wash"	1/2 h.			
BENSCH e.a. 1967	-	dog	I^{131}-albumin I^{131}-globulin	intratracheal instillation	15 m. à 7 d.			
DOMINGUEZ e.a. 67	-	guinea pig dog	albumin polyvinylpyrrolidone	intratracheal injection	1/2 à 48h à 8 d.	+ +		
NIDEN, 1967	+ (EM)	mouse	carbon	inhalation	1/2 h.			
SANDERS and ADEE, 1968	+ (LO+ EM)	rat	polonium-210	inhalation			+	

TYPE II CELL	TYPE I CELL	EPITH. JUNCT.	INTER-STITIUM	CONN. TISSUE	END. BV.	END. BV. JUNCT.	LUM.BV.	ENDOTH. LYMPH.	END. L. JUNCT.	LUM. LYM.	LYM. NODE
							+				
							+			?*	
			+								
-	-										
epithelial cells			+	-							
+			+	-							
+			+	-							
+			+	-							
+			+	-							+
-	-										
±											
	+			+							
							+				
							+				
epithelial cells											
?*			+	+						+	+
epithelial cells											
+											
epithelial cells											
+			+				+			+	
+			+				+			+	
							+				
+											
							+				
							+				
+											
	+										

SUZUKI e.a. 1968	+ (EM)	hamster	asbestos fibers	intratracheal instillation	1 à 16 d.		+	
FAULKNER and ESTERLY, 1969	+ (EM)	rabbit (adult+ neonat.)	india ink	intratracheal injection	45 à 60 m.		+	+
ER e.a. 1969	-	dog	albumin-I^{131}	intratracheal instillation	10 m. à 4 h.			
SUZUKI and CHURG, 1969	+ (EM)	hamster	asbestos fibers	intratracheal instillation	1 à 16 d. 6 à 24 mo.		+	+
CORRIN, 1970	+ (EM)	rat	carbon	intratracheal instillation	3 h. à 120h 3 h. à 48 h		+ +	
DERMER, 1970	+ (EM)	guinea pig	surfactant				+	
ESTERLY and FAULKNER, 1970	+ (EM)	rabbit	india ink polystyrene	intratracheal injection	45 à 60m. 45 à 60m.		+ +	
SANDERS, 1970	+ (EM)	hamster rat	PuO_2-239	inhalation	30 m. à 7 d.		+	
SANDERS and AD , 1970	+(LO+ EM)	rat	$^{239}PuO_2$	inhalation	30 m. à 7 d.		+	
BENSCH and DOMINGUEZ, 1971	+ (EM)	guinea pig	H.R.P.	intratracheal instillation	15 m. à 4 h.		+	
SANDERS e.a. 71	+ (EM)	hamster	NiO Cr_2O_3	inhalation inhalation	3 d. 3 d.		+ +	
SCHNEEBERGER and KARNOVSKY 1971	+ (EM)	neonatal mouse	H.R.P.	intranasal instillation	20 à 60m.			
GONZALEZ-CRUSSI and BOSTON, 1972	+ (EM)	fetal rabbit at term.	H.R.P.	intratracheal instillation	30 m. à 120 m.			

LEGEND

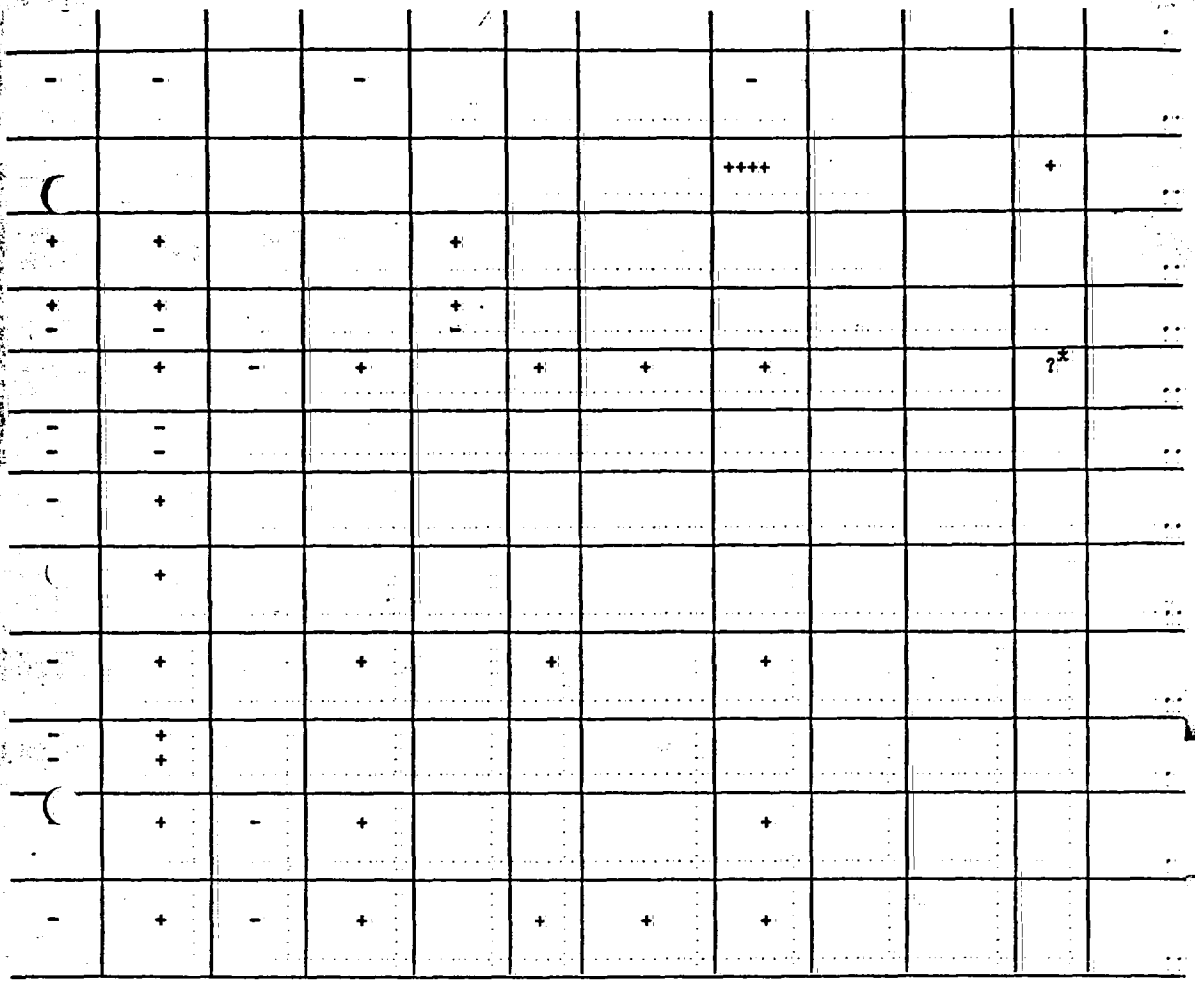
Morph. Invest. : Morphological Investigation

Time Int. : Time Interval (h. : hours; m. : minutes; mo. : months; d. : days).

P. Alv. Wall : Permeability of the Alveolar Wall

Alv. Macr. : Alveolar Macrophage

Leukoc. : Leukocyte



LEGEND

Type II Cell : Type II alveolar epithelial Cell

Type I Cell : Type I alveolar epithelial Cell

Epith. Junct. : Epithelial Junction

Interstitium : Basement Membrane and Interstitium

Conn. Tissue : Connective Tissue Cell

End. BV. : Endothelial Cells of Blood Vessels

End. BV. Junct. : Endothelial Junctions of Blood Vessels

Lum. BV. : Lumen of Blood Vessels

Endoth. Lymph. : Endothelial Cells of Lymphatics

End. L. Junct. : Endothelial Junctions of Lymphatics

Lum. Lym. : Lumen of Lymphatics

Lym. Node : Lymph Node

?* : not demonstrated ; hypothetically formulated

About one year ago (see progress report -) we have started an experimental study in this respect (using ferritin as a tracer), and it is obvious (Lauweryns and collaborators, unpublished data) that a careful experimental investigation using various morphological techniques and considering the newest literature data on lung structure and function, will yield new and basic results.

1.3. As regards morphological studies of the lung lymphatics in various pathological conditions of the lung parenchyma, it may be stated that no real data are available in the literature, except for casual annotations and our earlier histological (Lauweryns et al., publication nr. (38), 1965, (39), 1965) and morphometrical (Lauweryns et al., publication nr. (56), 1968, (80), 1969) studies on the lung lymphatics in neonatal hyaline membrane disease (76), 1968, (88), 1970) and in drowning (Lauweryns, (92), 1970).

Still the formation of lung edema occurs daily in medical practice in a large number of patients, in association e.g. with chronic and progressive respiratory insufficiency as in chronic bronchitis or emphysema, shock lungs (cardiogenic and neurogenic), uremia, drowning...

It does not seem believable that (almost) nothing is known about the fine morphology of the lung lymphatics in diseased and edematous lungs.

(2) Aims and rationale

From the foregoing we feel that our studies on the lymphatics of the lung should be carried on, as they are not only basically important but are also directed to understand the diseased lung better.

The aims of the proposed study are indeed :

2.1. A further and thorough morphological investigation of the lymphatic system of normal lungs, i.e.

- High power electron microscopy and enzyme digestion studies of anchoring and endothelial filaments.
- Comparative electron microscopic investigations of capillaries, collecting ducts and conducting channels in various animal species and body localizations.
- Electron microscopic and scanning electron microscopic studies of the "roots" of the pulmonary lymphatic system and the valves.
- The innervation of the lung lymphatics.

2.2. An experimental approach of the various morphological factors (especially the lymphatics) involved in the clearance and drainage of the lung parenchyma of airborne particulate matter.

2.3. A morphological study of the lung lymphatics in lung edema and in various diseases (chronic progressive respiratory insufficiency as in chronic bronchitis or emphysema, shock lungs (cardiogenic and neurogenic), uremia, drowning.

These three area 2.1., 2.2. and 2.3. will be investigated in parallel during the three years of our research proposal.

2.1. Morphology of the pulmonary lymphatic system

The most urgent questions to be solved in this field are :

2.1.a. The nature and the function of the anchoring filaments.

As it is up to now virtually impossible to isolate these filaments and to submit them to chemical analysis, their nature and function might perhaps be discovered by a high resolution EM in order to compare them with other well known filaments like actin, myosin, protocollagen, combined with enzyme (hyaluronidase, elastase,..) digestion studies. One may also wonder about the nature and the function of the filaments situated within the lymphatic endothelial cells (Cecio, 1967). In a similar way high power electron microscopy for comparative reasons combined with enzyme digestion methods and the study of their morphological reaction towards pharmacological agents might reveal their nature and function.

2.1.b. The nature and the function of the very recently discovered pericentriolar filamentous bundles (Lauweryns et al., (124), (125), in press). A study of their distribution in different cell types and in various animal species might at least reveal wether they are specific to some animals, to some cell types and might hence suggest possible roles. If they were specific to lymphatic endothelium and present in various species, their role would certainly be related to lymphatic function.

2.1.c. The endings of the pulmonary lymphatic system. Are the terminal divisions of the small lymphatic capillaries (the initial lymphatics, Casley-Smith, 1961) blind fingerlike projections, on themselves closed loops, or is there fine communication with tissue clefts (Klika, 1968) ? This problem can best be studied by the use of electron microscopic techniques which allow a certain degree of tridimensional visualization and surface view, i.e. freeze-etching and especially scanning electron microscopy.

2.1.d. The pulmonary lymphatic valves. Our graphic reconstructions (Lauweryns et al., publication nr. (85), 1970, (86), 1970, (89), 1971, (96), 1971, (121), 1972) and stereomicroscopic observations (Lauweryns, publication nr. (111), 1971, (115), 1971) of pulmonary lymphatic valves could most adequately be confirmed and extended by scanning electron microscopy of these valves.

2.1.e. The morphological differences between lymphatic capillaries, collecting channels and conducting channels. It is hardly believable that no data are known concerning this subject. The answer to this question implies a thorough electron microscopical and histological observation of a large number of lymphatics in various localizations and in various animals.

2.1.f. The innervation of the lung lymphatics and their valves. (Gellert, 1967, Kubik, 1955, Melnikova, 1964, Schipp, 1965, Shdanow, 1967, Vajda, 1966). Here a combined neurohistochemical and electron microscopic investigation - as we have executed in other areas (Lauweryns et al., publication nr. (84), 1969, (106), 1970, (108), 1971, (117), 1972) - will lead to an answer.

Rationale : These various morphological problems are to some extent related and can also only be accurately solved (Lauweryns et al., publication nr. (86), 1969, (96), 1971) by a combined and multidisciplinary approach including various techniques, which are familiar to us, i.e. : - anatomical injection studies, - serial reconstructions, - radiography and microradiography, - histological techniques, - morphometrical studies, - histochemical techniques and - electron microscopy.

All these techniques currently used at our laboratory have indeed their limitations, and some of them could even produce artefacts. Injection of the lymphatics of the pleura i.e. with radioopaque substances followed by radiography, or with plastic substances followed by corrosion of the tissues, easily causes disruption of the delicate walls of the smaller lymphatic vessels and filling of tissue clefts. The injection moreover may inverse valves and hence result in false pictures of lymphatic drainage-pathways. Corrosion casts and radiographs moreover do not reveal the relationship of the vessels to the surrounding tissues in fine detail.

Histological examination i.e. of non injected lymphatics is hindered by difficulties in recognizing pulmonary lymphatic capillaries and in differentiating them with certitude from smallblood vessels and tissue clefts. Histochemical methods to differentiate lymphatic vessels from other structures are not known.

Reconstructions from serial histological sections (Staubesand et al., 1953, Comparini et al., 1965, Boussauw et al., publication nr. (85), 1970) are subject to the same difficulties and have moreover the disadvantage of being a time consuming procedure.

Electronmicroscopy is also limited because only very small areas can be investigated.

Freeze-etching electron microscopy will also be applied. This technique is different from traditional electron microscopy because it avoids some chemical interactions in the tissues, exposes tissue structures in relief and allows the observation of membrane surfaces "en face". We are thoroughly acquainted with

this technique, having already done extensive studies on the lung parenchyma (Lauweryns et al., publication nr. (93), 1971, (97), 1970, (100), 1971, (128), submitted). In studying the lymphatics, it still poses one major problem as basal membranes are not visible on freeze-etch electron micrographs (hence hindering the differentiation from lymphatic capillaries with other small vessels or even tissue clefts).

Scanning electron microscopy will also be executed. It will certainly add important information and new, original and unique data to our field of study ; indeed it allows the investigation of relatively large samples (as compared to transmission electron microscopy) both at low and higher magnification and with great focal depth. Moreover the peculiar tridimensional "geometrical" structure of the lung seems to be ideally adapted for SEM investigation.

From the limitations and inherent risks of each of these methods, we feel that only a multidisciplinary investigation by applying various methods of investigation can result in a clear, synthetic and true picture of the pulmonary lymphatic system, as already stands out from our earlier work (Lauweryns et al., publication nr. (26), 1962, (31), 1963, (34), 1964, (36), 1964, (37), 1965, (38), 1965, (39), 1965, (46), 1966, (56), 1968, (57), 1968, (60), 1968, (69), 1970, (70), 1968, (74), 1968, (76), 1968, (80), 1969, (84), 1969, (85), 1969, (86), 1970, (88), 1970, (89), 1971, (96), 1971, (104), 1970, (106), 1970, (108), 1971, (111), 1971, (115), 1971, (117), 1972, (121), 1972, (124) & (125) in press).

2.2. Experimental study of the various morphological factors (especially the lymphatics) involved in fluid transport and clearance of particulate matter

Following the pathway(s) which free particles have to follow to reach the lymphatic capillary lumen from the alveolar lumen, the following factors have to be considered : - alveolar macrophages, - alveolar epithelium, - basal membrane(s), - alveolar interstitium with blood capillaries, - lymphatic endothelium.

2.2.a. The alveolar macrophages, whose origin is seriously controversed (Bertalauffy and Leblond, 1953, Karrer, 1960, Bowden et al., 1968, Pinketi et al., 1966) do represent an important factor in alveolar clearance (Karrer, 1958, Policard et al., 1959, Faulkner and Esterly, 1969, Sanders and Adee, 1970). They are sometimes aided in their task by leucocytes which may be present in the alveolar lumen as free cells (Gross and Westrick, 1954, Gieseking, 1958, Karrer, 1960, Suzuki and Churg, 1969, Faulkner and Esterly, 1969). After resorption of foreign material the macrophages may (Cole, 1944, Vorwald, 1950, Karrer, 1960, Davies, 1963) or may not (Gross and Hatch, 1962, Hatch and Gross, 1964, Casarett and Milley, 1964) migrate to the interstitium and further on to the lymphatics.

These different aspects should be investigated.

2.2.b. The phagocytotic properties of the alveolar epithelium. - As clearly demonstrated in table 1, extreme contradictions exist in the literature concerning the phagocytotic properties of the alveolar epithelium ; this problem is not only important as such, but also because it remains related to the cellular origin of the surfactant (Macklin, 1954, Kikkawa et al., 1965, Niden, 1967, Kuhn, 1968, Azzopardi and Thurlbeck, 1969, Dermer, 1970 a).

2.2.c. The permeability of the basal membrane(s) which are not observed on freeze-etching electron micrographs (Lauweryns et al, publication nr. (93), 1971, (97), 1971, (100), 1971, (128) submitted ; Friederici, 1968), also remains an open question (Schneeberger-Keeley and Karnovsky, 1968).

Precise studies with high resolution electron microscopy on the fate and migration of particles through the interstitium and their interaction with the connective tissue cells have also not been executed.

2.2.d. The way(s) by which particles enter the lymphatic lumen is mainly speculative (Heppleston, 1963). An intercellular transport via typical open junctions is usually accepted (Lauweryns et al., publication nr. (73), 1969), still a transcellular transport may occur as well (Casley-SMith, 1965 ; Leak and Burke, 1968).

It is also unsettled if the lymphatic endothelial lining cells are capable of storing, digesting or transporting particles ?

The same question may be asked concerning the blood capillaries.

Though both vessel systems (blood- and lymph vessels) probably play a role in alveolar clearance, their relative importance remains unsettled (Meyer et al., 1969, Dermer, 1970 b, Leak, 1970,1971).

Rationale : Though using in general terms the same spectrum of techniques, as mentioned under 2.1., this investigation will be mainly electron microscopical.

2.3. The lung lymphatics in various diseases

Being thoroughly acquainted with the lymphatics in the normal adult and infant lung and having approached them already in two diseases (i.e. Hyaline Membrane Disease of the human newborn (Lauweryns et al., publication nr. (38), 1965, (39), 1965, (56), 1968, (76), 1968, (80), 1969, (88), 1970) and drowning (Lauweryns, publication nr. (92), 1970), we propose to investigate them in various diseases (chronic and progressive respiratory insufficiency as in chronic bronchitis or emphysema, shock lungs (cardiogenic and neurogenic), uremia, drowning, hyaline membrane disease, lungs of other neonatal deaths), especially when these are accompanied with edema formation.

As practically nothing is known about this subject, it will remain a delicate task and constitute quite an endeavour ; it is however obvious that we are ideally prepared to investigate this challenging problem, being thoroughly acquainted with the normal anatomy of the lymphatics and being trained as a pathologist.

Rationale : Gross (including lymphatic injections) and routine microscopic studies (including morphometric pilot studies) of the lungs will be first executed. Depending on the obtained results further investigations (transmission electron microscopical, scanning electron microscopical, freeze-etching) will be furthermore undertaken.

B. METHODS OF PROCEDURE

(1) Species to be investigated

1.1. As regards the normal morphology of the pulmonary lymphatic system :

- rabbit (newborn and adult).
- human infant and adult lungs.

We are thoroughly acquainted with rabbit and human lungs, as we are studying them since 1958 (cfr. curriculum vitae).

1.2. As regards the experimental study :

- newborn rabbits.

We are indeed not only thoroughly acquainted with their lungs, but have already undertaken these experiments since one year with satisfactory results.

1.3. As regards the lung lymphatics in various diseases :

Their will be no problem of collecting lungs as we have currently about 120 infant and 350 adult postmortems yearly.

As regards electron microscopy, we have no problem to be immediately alerted and do the fixations as soon as possible ; we are indeed immediately informed by the "Newborn premature center", the "High care unit" and the "Clinic for chronic lung diseases". The efficiency of this delicate cooperation has been demonstrated in our earlier electron microscopical studies of "Hyaline Membrane Disease" and human "Lung Lymphatics" in normal lungs (cfr. publications - curriculum vitae).

It is clear from the above that we will have no problems to harvest the lungs needed for our investigation.

(2) Techniques to be applied to :

2.1. Normal morphology of the pulmonary lymphatic system :

The techniques to be applied are all familiar to us (enzyme digestion excepted). They are especially :

- high power electron microscopy (problems 2.1.a ; 2.1.b ; 2.1.e ; 2.1.f).
- high power electron microscopy and enzyme digestion (problems 2.1.a ; 2.1.b ; 2.1.e ; 2.1.f).
- freeze-etching electron microscopy (problems 2.1.d ; 2.1.c).
- scanning electron microscopy (problems 2.1.d ; 2.1.c).
- electron microscopy (problems 2.1.b ; 2.1.e).

As regards the innervation of the pulmonary lymphatics, this will be studied by the classical histological techniques of silver impregnation (Bodian-Van Campenhout), by histochemical studies on cholinesterases (method of Koelle, modified by Gerebtzoff (1959) and by freeze-drying-fluorescent studies on catecholamines (method of Falck, 1962, 1965). We are thoroughly acquainted with these techniques (cfr. Lauweryns et al., publication nr. (59), 1967, (64), 1969, (84), 1969, (106), 1970, (117), 1972).

For a distinction of cholinergic and adrenergic nerve endings, we will apply the methods of Richardson (1966), De Robertis and Pellegrino, Wood and Barnett, (1964). Here also high resolution electron microscopy is best applied.

2.2. Experimental study :

- This will be the continuation of our study, started about one year ago. Under slight anesthesia, a tracer (0,05 cc) will be intratracheally instillated. Instillation causes indeed much less tissue disturbance or damage than injection. The amount of tracer introduced in the lungs by this procedure is also constant ; this is not true in cases of intranasal instillation.

- The animals are killed at various intervals after the instillation (from 15 min. to 24 days) and a careful electron microscopical investigation of both lungs carried out.

- Morphometric studies will be carried out to estimate the removal of the tracer and its fine localization in the lung.

- As tracer we will first use a colloidal solution of ferritin (Ø 100 à 110 Å, MW ± 465000, 2 X cristalline, cadmium free, N.B.C., Cleveland) ; Indeed ferritin is not only a widely used and efficiant tracer (biological protein, electrondense, non-toxic, Bruns and Palade, 1968) but has also never been used in an analogous study.

- To check the probable influence of the physico-chemical properties of the tracer ferritin, we will next use a totally different tracer, i.e. a carbon suspension (C11/1431 a; Günther Wagner, Hanover) whose composition has been described by Biozzi et al., (1953).

- Both series of results will be compared.

- After these studies using ferritin and a carbon suspension, other tracers could be used, if necessary (i.e. horseradish peroxidase ; thorotrast ; colloidal gold ; etc).

2.3. The lung lymphatics in various diseases :

As this constitutes an entirely new investigation with no reliable literature data available, we will apply the whole spectrum of morphological techniques which we have used earlier to study the lymphatics in normal lungs. Indeed and as explained in Rationale 2.1., each technique having its shortcomings, a combined and multidisciplinary approach is inevitable including various methods, which are familiar to us : - anatomical injection studies, - serial reconstructions, - radiography and microradiography, - histological techniques, -morphometrical techniques, - histochemical techniques, - transmission electron microscopy, - freeze-etching electron microscopy and scanning electron microscopy.

The "pilot approach" will include detailed gross and microscopic studies, combined with some injection studies and elementary morphometry.

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ADDENDUM 2

9. SEPARATE LIST OF PHYSICAL FACILITIES AVAILABLE

ADDENDUM 2

List of physical facilities available

a) for histology :
- all routine equipment : drying-stoves, rotary microtome Spencer, microtome for large sections Tetrander Jung, 3 binocular microscopes, 2 stereomicroscopes.

b) for radiography :
- radiographical apparatus Balteau with image intensifier.
- microradiographical apparatus Baltodyne, Balteau-General Electric.

c) for histochemistry :
- cryostat Pearce.
- freeze-drying apparatus Pearce.

d) for morphometry :
- one microscope with lateral projection arm.
- one Olivetti calculator.

e) for electron microscopy :
- one ultramicrotome Porter Blum MT-2.
- one automatic ultramicrotome Reichert.
- one vacuum-coating apparatus Edwards.
- dack room facilities.
- one freeze-etching ultramicrotome Balzers.
- one electron microscope Zeiss Em 9 (resolution ± 12 Å)
- one electron microscope Philips EM 300 (resolution ± 3 Å).

ADDENDUM 3

10. ADDITIONAL REQUIREMENTS

ADDENDUM 3. Item 10, ADDITIONAL REQUIREMENTS

We have no scanning electron microscope available at our laboratory, but have been thoroughly introduced to this technique and discipline and carried out some preliminary work at the Faculty of Sciences and Engineering at the University of Ghent. However, the submitted research program in Scanning Electron Microscopy far exceeds the "occasional" possibilities we have to work in Ghent, which is furthermore situated at a distance of about 100 miles from Leuven (hence hindering considerably the efficiency of our work).

Moreover we cannot carry out an ideal preparation of our tissues at Ghent as we are not allowed there to execute a "critical point drying" of the lung tissues (which is however best and necessary for Scanning Electron Microscopy), but only "air drying" which badly cracks the lung tissues, causes many artefacts and produces very often poor results. To investigate the ultrastructure of the delicate lung lymphatics and obtain reliable results, it is absolutely necessary to have a Scanning Electron Microscope continuously available at the laboratory to study the specimens without artificially induced artefacts.

Hence we have contacted the Philips Company who has offered us the "Philips PSEM - 500 Quantitative High Performance Scanning Microscope", cost price (tax included) : $ 106,296. They have allowed us to pay the instrument in three yearly payments without rent on the due sums in the meantime.

We have then contacted our local university, which will consider to intervene for 50% in the cost price, if CTR first approves our research proposal.

Hence the payments will be as follows :

i.e. : - first year : one third : $ 35.432 : - CTR : $ 17,716 (one sixth)
- University of Leuven : $ 17,716 (one sixth)

- second year : one third : $ 35.432 : - CTR : $ 17,716
- University of Leuven : $ 17,716

- third year : one third : $ 35.432 : - CTR : $ 17,716
- University of Leuven : $ 17,716

The price includes the installation in our laboratory. We have given the preference to the Philips instrument because we have already a Philips transmission electron microscope and hence know the excellent local maintenance service of this company in Belgium. Moreover the main factory is in Holland, hence very close to our laboratory, if a problem should arise. The same cannot be said in our particular case for other companies, which - as far as scanning electron microscopy is concerned - only have a local sale representation but no real technical service available.